

AD_____

Award Number: W81XWH-07-1-0531

TITLE: Discovery and Role of a Novel Eicosanoid Family in Breast Cancer

PRINCIPAL INVESTIGATOR: Claus Schneider, Ph.D.

CONTRACTING ORGANIZATION: Vanderbilt University Medical Center
Nashville, TN 37203-6869

REPORT DATE: August 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-08-2008		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 1 AUG 2007 - 31 JUL 2008	
4. TITLE AND SUBTITLE Discovery and Role of a Novel Eicosanoid Family in Breast Cancer				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-07-1-0531	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Claus Schneider, Ph.D. E-Mail: claus.schneider@vanderbilt.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Vanderbilt University Medical Center Nashville, TN 37203-6869				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT There is strong evidence for the induction of the pro-inflammatory enzymes 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) in many types of cancer, including breast cancer. Upregulation of both enzymes is associated with promoting tumorigenesis and a negative prognosis of the disease. The 5-LOX (leukotriene) and COX-2 (prostaglandin) pathways have traditionally been viewed as independent biosynthetic routes to eicosanoid lipid hormones, yet recent enzymological analyses establish that the 5-LOX product, 5-HETE, is an excellent COX-2 substrate, forming a novel double endoperoxide product that has the potential to evolve into a novel family of biomediators. In this funding period we have used LC-MS to detect two cellular metabolites that were derived from the novel di-endoperoxide product. The metabolites are similar in molecular weight to the parent di-endoperoxide, and their formation is blocked by presence of the COX-2 inhibitor indomethacin. Detection of these products is a major step forward in establishing a potential novel eicosanoid-forming pathway resulting from the cross-over of the leukotriene and prostaglandin pathways in breast cancer.					
15. SUBJECT TERMS Eicosanoid, COX-2, 5-lipoxygenase, arachidonic acid					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER (include area code)

Introduction

There is strong evidence for the induction of the pro-inflammatory enzymes 5-lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) in many types of cancer, including breast cancer. Upregulation of either enzyme is associated with promoting tumorigenesis and a negative prognosis of the disease. The 5-LOX (leukotriene) and COX-2 (prostaglandin) pathways have traditionally been viewed as independent biosynthetic routes to eicosanoid lipid hormones because either enzyme catalyzes the initiating transformation of arachidonic acid toward their respective pathway. Yet our recent enzymological analyses established that the 5-LOX product, 5-HETE, is an excellent substrate for COX-2, forming a novel di-endoperoxide product [1]; this di-endoperoxide product has the potential to evolve into a novel family of highly oxygenated eicosanoid biomediators. In this project we tested the hypothesis that novel eicosanoids derived from the cross-over of the 5-LOX and COX-2 pathways can be detected in breast cancer cells that are positive for both enzymes. The long-term goal of this project is to investigate how these novel eicosanoids regulate proliferation and differentiation of breast cancer cells, and thus, to elucidate whether pharmacological manipulation of formation of these novel eicosanoids can provide a strategy for the treatment breast cancer.

Body

Task 1. To investigate the expression of 5-LOX and COX-2 in breast cancer cell lines

When we initiated work on this task we found that the commercially available antibodies for 5-LOX gave inconsistent and partially false positive signals (Fig. 1). We decided to generate our own peptide-derived polyclonal antibody for 5-LOX through a commercial outside contractor. The new antibody is quite sensitive and highly specific with no cross-reaction to the other five LOX isoforms. We have now acquired a range of different human breast cancer cell lines and are beginning the screen for expression of 5-LOX and COX-2 using Western blotting.

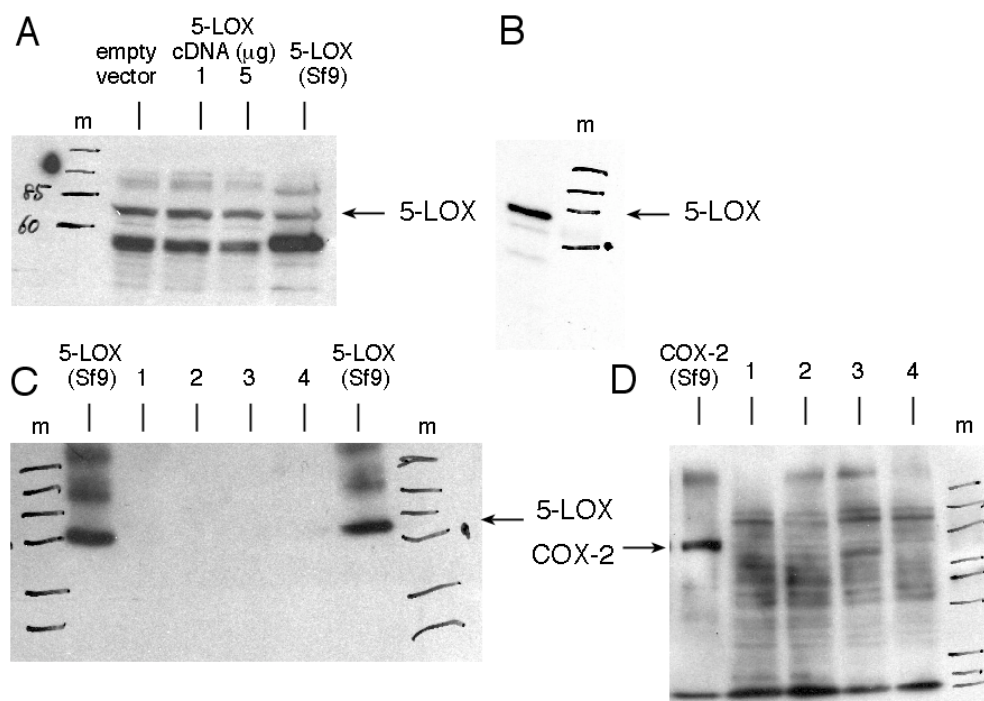


Fig. 1. Western blot detection of 5-LOX and COX-2. (A) An antibody for 5-LOX obtained from BD Biosciences detects a band that co-migrates with 5-LOX in COS-7 cells transfected with empty vector (negative control!), with 5-LOX cDNA at 1 and 5 μ g, and 5-LOX expressed in Sf9 insect cells. (B) We have generated a peptide antibody that is specific for detection of human 5-LOX in Western blotting. (C) Using the antibody shown in (B) we did not detect expression of 5-LOX in H2122 (lane 1), MCF-7 (lane 2), MDA-MB-231 (lane 3), or HCA7 cells (lane 4). (D) Expression of COX-2 was not detected in H2122, MCF-7, MDA-MB-231, or HCA7 cells (same loading order as in (C)).

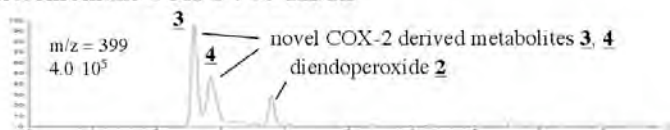
So far, we have not succeeded in detecting expression of either 5-LOX or COX-2 in the breast cancer (MCF-7, MDA-MB-231) or other cancer cell lines (H2122, HCA7) that we have tested.

Task 2. To investigate the formation of novel 5-LOX/COX-2 dependent metabolites in breast cancer cell lines

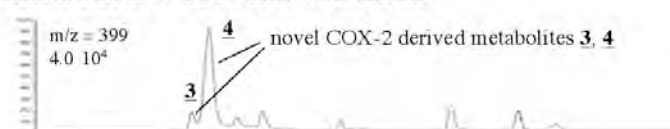
We investigated the transformation of 5-HETE in our current model of RAW264.7 macrophages that express high levels of COX-2 upon activation with LPS/IFN- γ . We prepared 5-HETE substrate labeled with deuterium at carbons 5,6,8,9,11,12,14,15 starting from d_8 -arachidonic acid and incubation with recombinant 5-lipoxygenase expressed in Sf9 insect cells.

Using d_8 -labeled 5-HETE **1** we observed formation of the previously identified di-endoperoxide **2** and two novel eicosanoids **3** and **4** when using recombinant human COX-2 (Fig. 2, top panel). Incubation of 5-HETE with activated RAW264.7 cells gave the same three products, albeit the ratio of products **3** to **4** was changed such that **4** was the more prominent product in the cellular incubations (Fig. 2, middle panel). The isotopic pattern clearly indicated that these products were derived from 5-HETE, but whereas the di-endoperoxide **2** retained all 8 deuterium labels, the novel metabolites **3** and **4** retained only 6 deuterium atoms and, therefore, have lost 2 labels during the transformation. LC-MS analyses showed that the molecular weight of **3** and **4** is the same as the parent di-endoperoxide **2** (MW = 400; Fig. 2), and UV analyses indicate the presence of a substituted keto-ene moiety. The di-endoperoxide **2** as well as the novel metabolites **3** and **4** were absent in cells treated with the non-selective COX inhibitor indomethacin, and they were also absent in unstimulated cells (Fig. 2, bottom panel).

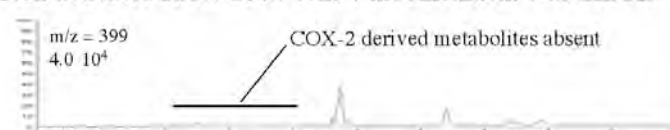
Recombinant COX-2 + 5S-HETE



Activated RAW 264.7 cells + 5S-HETE



Non-activated RAW 264.7 cells + indomethacin + 5S-HETE



Western blot

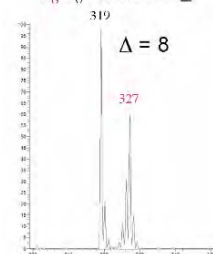
+ LPS/IFN- γ

COX-2

- LPS/IFN- γ

COX-2

d_8/d_0 -5S-HETE **1**



d_6/d_0 -labeled metabolite **4**

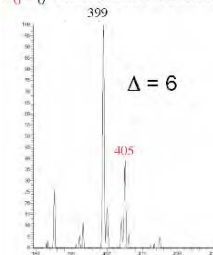


Fig. 2. LC-MS analyses of the transformation of 5-HETE by recombinant COX-2 (top panel), activated RAW264.7 cells (middle), and unactivated RAW264.7 cells in the presence of indomethacin (bottom). The ion traces for m/z 399 (negative ion mode) are shown at the same scale. On the right: LC-MS spectra of an approximate 1:1 mixture of d_0 and d_8 5-HETE incubated with activated RAW264.7 cells show that metabolites **4** and **3** (not shown) have lost 2 deuterium labels during the transformation. (The additional signals at m/z 326 and 325 in the d_0/d_8 5-HETE are derived from the substrate that was contaminated significantly with the d_7 and d_6 isomers.)

The results clearly indicate that the novel eicosanoids **3** and **4** are derived from conversion of 5-HETE **1** by COX-2, and that they are cellular transformation products of the di-endoperoxide **2** that we have previously characterized in vitro [1]. We are now in the process of isolating and structurally identifying eicosanoids **3** and **4**.

Task 3. To treat breast cancer cell lines with the novel metabolite and characterize its effect on differentiation, proliferation, and angiogenesis

This task will be addressed during the 12-month no cost extension period from August 1, 2008 – July 31, 2009.

Key Research Accomplishments

- A new antibody for detection of human 5-LOX was designed and generated.
- Two novel eicosanoids derived from the consecutive activities of 5-LOX and COX-2 were detected.

Reportable Outcomes

An abstract reporting the results of task 2 was presented at the AACR Special Conference on Chemical and Biological Aspects of Inflammation and Cancer (October 14-17, 2008) organized by the American Association for Cancer Research:

Markus Griesser, Takashi Suzuki, and Claus Schneider: "Characterization of novel 5-lipoxygenase/cyclooxygenase-2-derived eicosanoids". A copy of the abstract is attached as an appendix.

Conclusion

Our hypothesis of the cross-over of the 5-lipoxygenase and COX-2 pathways in breast cancer was supported by the detection of novel eicosanoids derived from the consecutive enzymatic conversion of arachidonic acid by both enzymes. Characterization of the biological activity of these novel mediators will answer the question whether pharmacological manipulation of this pathway can provide a strategy for the treatment of breast cancer.

References

[1] Schneider, C., Boeglin, W. E., Yin, H., Stec, D. F., Voehler, M., Convergent oxygenation of arachidonic acid by 5-lipoxygenase and cyclooxygenase-2. *J Am Chem Soc* 2006, 128, 720-721.

Appendices

A copy of the abstract presented at the AACR meeting is attached.

Characterization of novel 5-lipoxygenase/cyclooxygenase-2-derived eicosanoids

Markus Griesser, Takashi Suzuki, and Claus Schneider

Division of Clinical Pharmacology, Department of Pharmacology, Vanderbilt University
Medical Center, Nashville, TN 37232, USA

5-Lipoxygenase (5-LOX) and cyclooxygenase-2 (COX-2) initiate two separate biosynthetic pathways of lipid hormones (leukotrienes and prostaglandins, respectively) with distinct physiological roles in tissue homeostasis but also in inflammation and cancer. Both enzymes use arachidonic acid as the common substrate, and therefore the formation of the initial peroxide intermediate by 5-LOX or COX-2 is recognized as the committed step towards leukotriene or prostaglandin biosynthesis. We recently provided evidence for an unexpected biochemical interaction of the 5-LOX and COX-2 pathways: the 5-LOX product, 5S-hydroxyeicosatetraenoic acid (5S-HETE) is a selective and efficient substrate for the oxygenation by COX-2; the COX-1 isozyme does not react. *In vitro*, the major oxygenation product of 5S-HETE by COX-2 is a novel bicyclic di-endoperoxide. In activated RAW 264.7 cells a series of COX-2 derived metabolites of 5S-HETE was detected using LC-MS analyses. Isotopic labeling studies indicate that these eicosanoids were derived from the transformation of the intermediate di-endoperoxide. Further characterization of the cross-over of the 5-LOX and COX-2 pathways and its products could shed new light on the etiology and regulation of inflammatory diseases, and may also pave new ways for understanding the pathophysiology of other common diseases implicating 5-LOX and COX-2, like asthma and cancer.